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PHYSIOLOGY OF INSECT ECDYSIS. II. THE ASSAY AND OCCURRENCE OF THE ECLOSION HORMONE IN THE CHINESE OAK, SILKMOTH, *ANTHERAEA PERNYI*

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The transformation of the silkmoth pupa into the moth culminates with the emergence, or eclosion, of the adult from the old pupal skin. This event marks the "turning-on" of adult behavior and is accomplished through a series of distinct behavioral acts (Truman, 1971a; Truman and Sokolove, 1972). Early experiments which involved brain extirpation and transplantation suggested that eclosion was triggered by a neurosecretory hormone which was released by the brain (Truman and Riddiford, 1970). This inference was then confirmed by the demonstration of eclosion stimulating activity in homogenates of the silkmoth brain and corpora cardiaca (Truman and Riddiford, 1970; Truman, 1971a). The present study extends these preliminary observations. It describes in detail the assay for the eclosion hormone and also its appearance and distribution in the nervous system of the Chinese oak silkmoth, *Antheraea pernyi*.

Materials and Methods

1. *Experimental animals*

Diapausing pupae of *Antheraea pernyi* were obtained from Japanese dealers and stored at 5° C. When needed, pupae were brought to 26° C. Typically, adult development was initiated within a week, and adult emergence followed about 3 weeks later. Some experiments also utilized the tobacco hornworm, *Manduca sexta*. The origin and handling of these insects was as described in Truman (1972).

2. *The biological assay for the eclosion hormone*

In the Pernyi moth, the initiation of adult development is signalled by the retraction of the wing epidermis from the overlying pupal cuticle. Once initiated formation of the adult then requires 19 days at 26° C (Truman, 1970 as modified from Williams and Adkisson, 1964). At the end of this period one finds a "pharate moth"—a moth which has completed all overt signs of adult development but which is still encased in the pupal skin.

The eclosion hormone assay utilized pharate Pernyi moths. For at least one week prior to their use the developing moths were exposed to a defined photoperiod. Routinely, this was a 17L : 7D regimen (photophase from 07 : 00 to 24 :00). Under these conditions pharate specimens of Pernyi eclose only during a "gate" in the late evening which occurs between 18 : 30 and 23 : 30 (Truman, 1971b).

Tissues to be assayed for hormone activity were homogenized with a ground glass homogenizer in a small volume (usually 20 μl) of insect Ringer's (Ephrussi and Beadle, 1936). Homogenates were either assayed immediately or frozen until tested. Each sample was injected by means of a 50 μl Hamilton syringe into the mesothoracic tergum of an assay animal. Pharate Pernyi moths were injected approximately 10 hours before their anticipated eclosion gate. At this stage the surrounding pupal cuticle shows complete digestion of the endocuticle and the ecdysial seams rupture when gentle pressure is applied with the fingers.

The presence of eclosion hormone activity was indicated by eclosion 1.5 to 4.5 hours after injection. If the moth did not emerge until its normal gate that same evening, the assay was scored negative. Occasionally a moth would not emerge on the day of injection but, rather, during its gate on the following day. In such cases it was assumed that the moth was not competent to respond at the time of injection and the assay was discarded.

Results

1. Quantitative aspects of the Pernyi assay

During the early stages of this study it was noted that extracts presumed to be rich in hormone (*i.e.*, from pharate moths) provoked eclosion more rapidly than did extracts thought to be poor in hormone (from newly emerged adults). Thus, it appeared that the latent period between injection and eclosion could provide a semi-quantitative estimate of the amount of hormone injected.

As seen below, the corpora cardiaca (CC) from early day-19 Pernyi were very potent sources of the ecolsion hormone. Therefore, these organs were removed from pharate moths at this stage, homogenized, diluted to various concentrations and injected into weighed test insects. The reciprocal of the latent period (the time between injection and eclosion) was taken as the score of the positive responses. Negative responses were scored as zero.

Figure 1 shows the results of 63 assays performed on corpora cardiaca-corpora allata (CC-CA) complexes obtained from day-19 pharate moths. The amount of hormone injected is expressed in corpora cardiaca equivalents per gram of assay animal (CC equiv/g). As seen in Figure 1, a dose of 0.15 CC equiv/g caused one-half of the assay animals to emerge. Beyond 0.30 CC equiv/g responses were almost exclusively positive.

The scores computed for the positive assays showed considerable scatter, but a clear dependence of score upon dose was observed. Table I gives the average scores computed for each 0.1 CC equiv/g increment in dosage. From concentrations of 0.05 to 0.60 CC equiv/g, the average score increased in a linear fashion to a value of 0.60. Concentrations greater than 0.60 CC equiv/g failed to produce higher average scores (Fig. 1).

The substantial scatter was probably due to individual variation in both the assay animals and the CC-CA complexes tested. The variation in the latter was underscored by a series of 17 assays performed on the pooled material from 9 CC-CA complexes (Fig. 1, open circles). The scores obtained with the pooled homogenate showed considerably less scatter than did the remainder of the assays which were each performed on single pairs of glands.

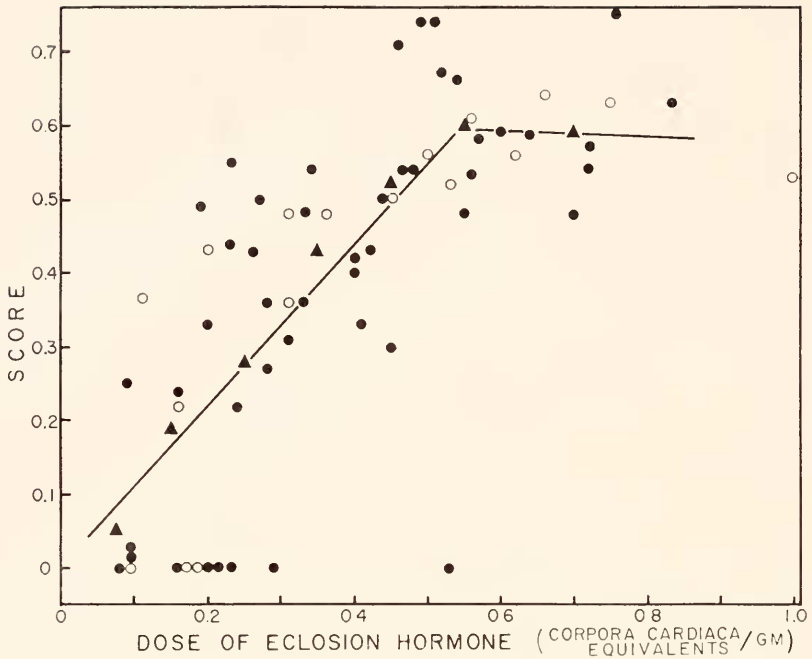

FIGURE 1. A dose-response curve for the eclosion hormone using the Peryni assay. The score of positive assays was computed as the reciprocal of the latent period, the time between injection of the hormone and emergence of the moth. Filled circles were tests performed on single CC-CA complexes. The open circles were tests performed on the pooled material from 9 complexes. The filled triangles are average scores obtained from Table I. The lines were obtained by the least squares method for the data from 0 to 0.6 and from 0.5 to 1.0 CC equiv/g respectively. The zero score at 0.53 CC equiv/g was omitted.

A score of 0.60 represents a latent period of approximately 1.7 hr. Of this time, the last 1.25 to 1.5 hr is required for the performance of the pre-eclosion behavior—a stereotyped piece of behavior which immediately precedes eclosion (Truman, 1971a). Therefore, the hormone injection triggers a change in overt

TABLE I

The effect of various doses of eclosion hormone containing homogenates in the Pernyi assay

Dosage (CC equiv/g)	No. of assays	Scores	Average score
0.05–0.10	5	0,0,0,0,0.25	0.05
0.11–0.20	9	0,0,0,0,0.22,0.24,0.33,0.43,0.49	0.19
0.21–0.30	10	0,0,0,0.22, 0.27, 0.36, 0.43, 0.44, 0.50, 0.55	0.28
0.31–0.40	9	0.31,0.36,0.36,0.40,0.42,0.48,0.48,0.48,0.54	0.43
0.41–0.50	10	0.30,0.33,0.43,0.50,0.50,0.54,0.54,0.56,0.71,0.74	0.52
0.51–0.60	10	0,0.48,0.56,0.53,0.58,0.59,0.61,0.66,0.67,0.74	0.60*
>0.60	10	0.48,0.53,0.54,0.56,0.57,0.58,0.63,0.63,0.64,0.74	0.59

* Zero score was considered to be a false negative assay and therefore was not included in the computation of the average score.

TABLE II

Distribution of eclosion hormone in the nervous system of day-19, pharate, Pernyi moths

Tissue	No. of assays	% Positive responses
Abdominal ganglia	5	0
Thoracic ganglia	5	0
Brain	10	60
Corpora allata-corpora cardiaca complex	9	100
Corpora allata	8	0
Corpora cardiaca	7	100
Body of CC	11	91
CC nerve leading to heart	10	60

behavior within 10 to 20 minutes. Presumably the time between hormone release and the onset of the pre-eclosion behavior in the intact animal is similar.

A pair of CC injected into a 3 g test animal is equivalent to a dose of 0.067 CC equiv/g or approximately 4.5 times the hormone required to give a 50% positive score. Thus, a day-19 pharate moth has stored 2 to 3 times the hormone necessary to trigger eclosion.

2. Distribution of eclosion hormone in the moth nervous system

Specimens of day-19 Pernyi were dissected. Various portions of the nervous system were excised and tested in the Pernyi assay. Table II shows that eclosion hormone activity was confined exclusively to the cephalic portion of the nervous system—homogenates prepared from the thoracic or abdominal ganglia consistently failed to cause eclosion.

In the head considerable activity was obtained from the brain—positive scores were obtained in 6 of 10 trials. But as seen in Table II, the highest activity was recovered from the CC-CA complex. In order to identify the source of activity in the complex, the CA were carefully dissected from the CC and the two glands assayed separately. In all assays the CC produced positive responses, whereas the CA had no effect.

The CC of silkmoths is a complicated structure which receives nerves from the brain and also sends a number of roots to the periphery. The largest nerve leading from the CC extends the short distance to the heart and ends in the heart wall. In 11 CC-CA complexes, the nerves leading to the heart were severed as close to the body of the CC as possible. The CC and the severed nerve including the section of heart into which it entered were then each tested. The CC consistently showed activity. Also, in many cases the nerves leading to the heart had substantial activity. Therefore, eclosion hormone may be transported from the CC to the heart wall in preparation for release. This arrangement would ensure the most rapid distribution of hormone through the moth.

3. Source of the eclosion hormone

When the corpora cardiaca are removed from Cecropia pupae, the neurohaemal portions of these structures rapidly regenerate, but the intrinsic functions of the

CC are lost (Stumm-Zollinger, 1957). The moths which result from such pupae, however, show a perfectly normal timing of eclosion (Truman and Riddiford, 1970), indicating a release of eclosion hormone at the time of adult emergence. Therefore, the high titers of activity observed in the CC undoubtedly arise from hormone which is produced in the brain and then is transported to the CC for storage and eventual release. In the moth brain the neurosecretory cells are found primarily in median and lateral cell clusters. Also, a few are located in a small

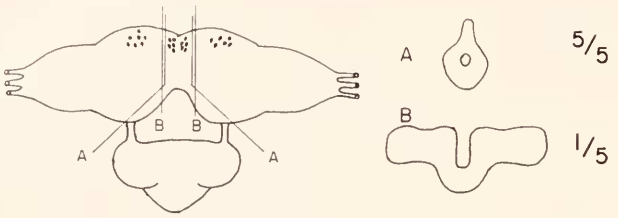

FIGURE 2. Left: schematic representation of the pupal brain showing the cuts made to isolate the median (A) or lateral (B) neurosecretory cells. In both cases the piece to be implanted was left attached to the subesophageal ganglion; Right: brain fragments after metamorphosis: (A) piece containing median neurosecretory cells; (B) piece containing lateral cells. The fraction shows the number of assays which were positive for the eclosion hormone.

posterior group (Herman and Gilbert, 1965). In order to determine which of the two major groups might be responsible for hormone production, brains from *Manduca* pupae were divided as shown in Figure 2. Hornworms were chosen for this experiment because both the median and lateral clusters of neurosecretory cells are clearly visible in the living brain when viewed against a black background under a dissecting microscope. The brain fragments were implanted into brainless hornworm pupae which were then caused to develop by injection of β-ecdysone. On the next to last day of adult development, the moths were sacrificed and the fragments recovered. As noted in Figure 2, pieces containing the median neurosecretory cell cluster had activity in 5 of 5 cases, whereas the lateral group gave a positive response in 1 of 5 assays. The positive response obtained in this latter group was presumably due to a few median neurosecretory cells being retained in a lateral piece. These data, therefore, indicate that the cells in the median neurosecretory cluster are probably responsible for production of the eclosion hormone.

4. Eclosion hormone activity in the brain and corpora cardiaca

Larval brains and corpora cardiaca. In tobacco hornworms, the fourth instar requires 2 to 3 days. The larvae then release prothoracicotropic hormone and, 50 hrs later, ecdyse to the 5th instar (Truman, 1972). Brains with attached CC were removed from pairs of *Manduca* larvae which were within 12 hr of the time of ecdysis to the 5th instar. As seen in Table III, no eclosion hormone activity was recovered from larvae at this stage.

During the feeding portion of the fifth instar, which lasts 4 to 5 days, tests on the brain and CC of individual larvae revealed little activity. Tissue removed during the first half of this period gave positive responses in only 1 of 7 assays.

Similarly, late 5th instar larvae did not yield any positive scores. This marginal level of activity continued through the cessation of feeding and the voiding of the gut (Table III).

Prepupal brains and corpora cardiaca. The onset of the prepupal period is signalled by retraction of the ocellar pigment and occurs approximately 1.5 days after the voiding of the gut. The brain and corpora cardiaca from the early prepupae showed only a very low level of activity. Even late prepupae, which had begun tanning of the underlying pupal cuticle, failed to yield substantial activity.

TABLE III

Amount of eclosion hormone activity found in brain and corpora cardiaca of tobacco hornworms at various stages in their life history

Stage	No. of assays	No. of positive scores	Average score
Pharate fifth instar larva	4*	0	0
Early fifth instar	7†	1	0.04
Late fifth instar	11†	0	0
Day of voiding gut	11†	1	0.02
Early prepupa	12†	2	0.04
Late prepupa	12†	1	0.02
Fresh pupa	13†	2	0.04
Pharate adult	12†	11	0.37

* Each assay utilized the brain and corpora cardiaca from two larvae.
† Each assay was performed on the brain and corpora cardiaca from a single donor.

Pupal brains and corpora cardiaca. Hornworm pupae were sacrificed within 24 hours after pupation and the brain and CC removed. As seen in Table III, hormonal activity in these structures was minimal.

Pharate adult brains and corpora cardiaca. Larvae of *Manduca* which had been reared under a 17L:7D photoperiod initiate adult development on the day following the pupal ecdysis. Seventeen days later development is complete and the moth emerges during that evening (M. M. Nijhout, Harvard University, unpublished). As is evident from Table III, pharate moths which were in early day 17 showed high concentrations of hormone in the brain and CC. This result will be considered in detail in the following section.

5. The titer of eclosion hormone during adult development of Pernyi moths

Between the initiation of adult development and eclosion, the developmental age of the Pernyi silkmoth can be estimated by features of the developing moth which can be seen through the pupal cuticle (Williams and Adkisson, 1964, as modified by Truman, 1970). Assays were performed separately on the brain and the CC from individuals at various stages in this development. Individual variation in both the donor and assay animals produced considerable scatter in the data, but some clear trends were evident.

Activity from the developing adult brain. A total of 79 assays were performed using homogenates prepared from brains of Pernyi pupae, developing adults, and adults. Figure 3A shows that activity could not be detected in single brains from

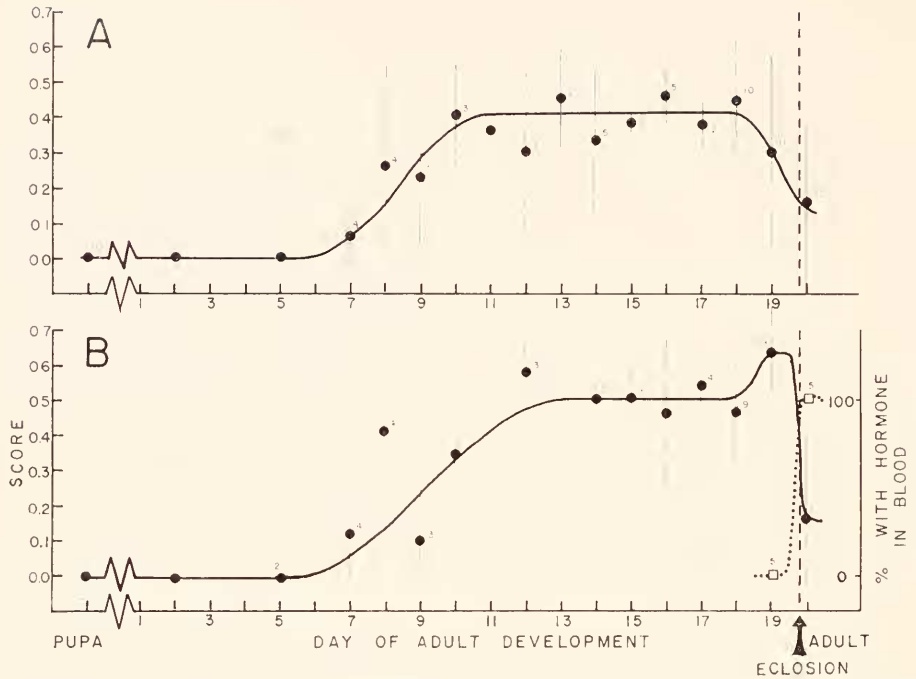

FIGURE 3. Changes in the eclosion hormone titer in the Pernyi silkworm as a function of the developmental age of the donor; (A) brain; (B) corpora cardiaca (●—●) and blood (□...□). The mean and standard deviation for each day are given. The number of animals assayed on each day is indicated.

Pernyi pupae or from developing adults up to day 7. On day 7, one of 4 brains tested showed low activity (score of 0.30). The average scores then increased through the next three days. Between day-10 and day-18, the brain titer appeared to remain relatively constant. There was a certain scatter in the data but the majority of the assays (69%) fell in the range between 0.30 and 0.50.

In a 17L:7D photoperiod, Pernyi emerge during the late evening of day-19. Therefore, it was surprising to find that on the morning of day-19, almost 12 hours before the time of eclosion, hormonal activity was disappearing from the moth brain (Fig. 3A). Of the 10 pharate moths which were sacrificed at this time, four gave negative assays.

This decline in activity was further evident in the adult. The brain of the newly emerged moth contained a level of hormone activity which was lower than any time during the preceding two weeks. Furthermore, 8 of 12 brains failed to show eclosion hormone activity.

Activity from the developing adult corpora cardiaca. During the pupal stage and the first week of adult development, the hormone titers in the CC mimicked those seen in the brain (Fig. 3B). Activity was first detected on day 7. The hormone concentration then increased until approximately day-12.

As with the brain, the amount of hormone in the CC appeared to remain rela-

tively constant between day-12 and day-18. During this period the average activity observed in the CC was consistently higher than that obtained from the brains from Pernyi moths of similar age.

As noted above, the hormone concentration in the brain begins to decline early on day-19. It is therefore of interest that the CC at this time reach their highest activity. Apparently, at this point in development, the brain is rapidly transporting the remaining hormone to the CC in preparation for its release that evening.

During the evening of day-19, the CC show a precipitous drop in hormone titer. These glands, excised from freshly emerged adults, produced an average score of only 0.17. Moreover, 6 of the 11 CC tested at this stage gave negative assays.

Activity from the haemolymph. Abdomens were severed from pharate or newly emerged Pernyi moths. Gentle pressure was applied to the fragments to express the haemolymph into a small vial which contained a few crystals of phenylthiourea (Williams, 1952). Approximately 200 to 500 μl of haemolymph was obtained from each animal in this manner. Samples which were contaminated with gut contents were immediately discarded. The blood from each moth was lyophilized and the resulting powder resuspended, as well as possible, in 50 μl of Ringer's. This syrupy mixture was then assayed as described under Methods.

Haemolymph collected from each of five pharate moths on the morning of day-19 was tested for eclosion hormone activity. All samples gave negative results (Fig. 3B). By contrast, blood collected form Pernyi moths during the performance of the pre-eclosion behavior or just after emergence that evening (prior to the initiation of wing inflation) provoked eclosion for all five samples tested. Therefore, the precipitous drop in hormone activity in the CC is mirrored by its appearance in the blood.

6. Test for activity in non-Saturniids

As shown in Table III, homogenates prepared from the brains and CC of pharate tobacco hornworms has marked activity in the Pernyi assay. Brains and CC from two hemimetabolous insects were also tested for the presence of the eclosion hormone. The tissues were dissected from pharate adults of either the cockroach, *Leucophaea maderae,* or the linden bug, *Pyrrhocoris apterus,* and tested in the usual manner. As seen in Table IV, no activity was recovered from either of these insects.

TABLE IV

Tests of brains and corpora cardiaca from non-lepidopteran species in the Pernyi eclosion hormone assay

Donor	No. of assays	No. of brains and CC-CA complexes per assay	% positive responses
Leucophaea maderae	1	1	0
	3	2	0
	1	3	0
Pyrrhocoris apterus	1	60	0

TABLE V

Tests of juvenile hormone, ecdysone and bursicon in the Pernyi eclosion hormone assay

Material tested	Dosage	No. of assays	% positive responses
C18 Cecropia juvenile hormone	0.2 μg	10	0
β-ecdysone	8 μg	10	0
Bursicon (abdominal nerve cord from *Manduca*)	1	10	0

7. Non-activity of other insect hormones in the Pernyi assay

The effects of C-18 Cecropia juvenile hormone and of β-ecdysone in the Pernyi assay are recorded in Table V. The former was injected in 10 μl of olive oil and the latter in 10% isopropanol solution. Both hormones were injected in physiological doses (Meyer, Hanzmann, Schneiderman, Gilbert, and Boyette, 1970; Williams, 1968) and both proved to be completely inactive.

Since release of the tanning hormone, bursicon, is associated with the act of eclosion (Cottrell, 1962; Fraenkel and Hsiao, 1962), it was of interest to test this hormone in the Pernyi assay. The abdominal nerve cord of pharate adult *Manduca sexta* is a potent source of bursicon (Truman, in preparation). Table V shows that homogenates prepared from pharate nerve cords were without activity.

DISCUSSION

Two neurosecretory hormones are associated with insect molting: the prothoracicotropic hormone (PTTH), which promotes the secretion of ecdysone by the prothoracic glands (Williams, 1952), and bursicon, which is responsible for the cuticular tanning of freshly emerged insects (Cottrell, 1962; Fraenkel and Hsiao, 1962, 1965). The data in Table V clearly show that the eclosion hormone is distinct from bursicon. Portions of the moth nervous system which contain bursicon show no eclosion hormone activity. Similarly, homogenates of the brain and CC of pharate adult hornworms will not provoke tanning (Truman, unpublished).

At present there is not conclusive proof that the eclosion hormone is distinct from PTTH. However, the failure to find significant quantities of eclosion hormone during larval life—a time when PTTH is known to be released—casts doubt on the hypothesis that the two hormones are identical. Therefore, from the available evidence, I suggest that the triggering of eclosion is the function of a previously unknown insect hormone rather than a new function for a described hormone.

The suggestion of the name "neurotropic ecdysis hormone" in our initial report of the hormonal control of emergence (Truman and Riddiford, 1970) suggested that this mechanism might be generally applicable to the control of all insect ecdyses. However, a recent study of larval molting in *Manduca* has pointed out a major difference in the control of larval ecdysis *versus* adult eclosion (Truman, 1972). Adult eclosion is a gated event which is triggered by a biological clock located in the moth brain (Truman and Riddiford, 1970). By contrast, larval ecdysis is

not gated but is developmentally triggered. At a given temperature it occurs a constant time after the initiation of the molting process by PTTH and ecdysone (Truman, 1972). Furthermore, the timing of larval ecdysis remains unaltered, even when all influence of the head is abolished by neck ligation (Truman, 1972).

The data presented here also argue against the hypothesis that the eclosion hormone triggers the larval-larval and/or the larval-pupal ecdysis. No individual from the developing fifth instar larva through to the pupal stage contained significant amounts of the eclosion hormone (average scores of 0 to 0.04). Even prior to an ecdysis, a time at which one would logically expect a build-up of titer in the CC, the hormone is practically absent.

From the dose-response curve given in Figure 1, one can estimate that in *Manduca* the background levels of hormonal activity seen during the larval and pupal stages (scores of 0 to 0.04) represent, at best, one-tenth of the material found in the pharate hornworm moth (score of 0.37). Similarly, the failure to find activity in single brains or CC of Pernyi pupae indicates that a similar relationship holds for this species. Thus, in both species, synthesis of the eclosion hormone occurs primarily during adult development.

Since in moths the eclosion hormone functions only during adult emergence and not during any of the preceding ecdyses, the failure to find activity in cockroaches or bugs is not surprising. The latter are hemimetabolous insects which do not have a pupal stage interposed between the feeding larva and the adult. Thus, in these insects the adult ecdysis differs little from the larval ecdyses. Therefore, one is presented with the intriguing possibility that the eclosion hormone may be confined only to the holometabolous orders of insects. The validity of this interpretation awaits further study.

The restriction of the appearance of the eclosion hormones to a specific portion of the moth life history allows for a preliminary identification of periods of synthesis, transport, and release of hormonal material. The study of Scharrer (1962) on the cockroach, *Leucophaea maderae,* clearly showed that neurosecretory material is transported down the neurosecretory axons from the brain to the CC. Similarly, in the moth, activity recovered from the brain presumably represents material either in the cell body or in transit down axons leading from the median neurosecretory cluster. Activity in the CC would then be due to the accumulation of the hormone in the axon endings.

In Pernyi, hormone synthesis by the median neurosecretory cells begins approximately on day-7. At this early stage hormone transport is also occurring as manifest in the recovery of activity in the CC. Hormone titers continue to increase in the brain until day-10, at which time a plateau is attained. This constant level of hormone activity could be due either to the rate of transport becoming equal to the rate of synthesis or to the stopping of both synthesis and transport. The continuing increase of activity in the CC would favor the former alternative through day-12. But the subsequent attainment of a plateau in the CC indicates that both hormone synthesis and hormone transport cease after day-12. This interpretation, of course, assumes that there is no breakdown of hormone in the axon terminals and that release only occurs after the completion of adult development. With the onset of day-19, hormone transport resumes and the brain essentially empties of activity. Presumably at this stage synthesis remains shut-off.

During the evening of day-19, the pharate moth secretes the eclosion hormone into the haemolymph. At this time approximately 75% of the hormonal material stored in the CC is released. The fact that hormone can be recovered from nerves which lead directly from the CC into the heart suggests that this may be the final pathway of hormone into the blood.

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Summary

1. A semi-quantitative biological assay for the eclosion hormone is described.

2. In the pharate moth hormonal activity is confined to the brain and the corpora cardiaca (CC).

3. During the life history of the moth, activity is practically absent from the brain and CC of larval and pupal stages and appears only in preparation for adult emergence.

4. The titer of eclosion hormone in the brain and CC of Pernyi moths was followed through adult development. In both structures activity first appeared on about day 7. Titers then increased for the next 3 to 5 days followed by a plateau. On the morning of the day of emergence, the titer in the brain fell and that in the CC correspondingly reached its highest level. At the time of eclosion in the evening, the CC titer then dropped and hormone appeared in the blood.

5. Tests on brains and CC from cockroaches and bugs failed to show activity.

6. It was concluded that the eclosion hormone is used only for the pupal-adult ecdysis and, thus, is probably restricted to the holometabolous orders of insects.

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